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Altered Gene Transcription After Burn Injury Results in Depressed T-Lymphocyte Activation

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Objective

Patients with major burns and an animal model of burn injury were studied to determine the mechanism of depressed interleukin-2 (IL-2) production after thermal injury and to determine the effect of such injury on IL-2 receptor (IL-2R) expression and function.

Summary Background Data

Major burn injury is known to diminish resistance to infection by altering cytokine production and prostanoic secretion and by inhibiting T-lymphocyte activation. T-cell activation requires production of regulatory cytokines, principally IL-2, and expression of the appropriate cytokine receptors. Depressed IL-2 production after major burn injury is undisputed, although the molecular mechanisms remain undefined; the effect of burn injury on IL-2R expression and function currently is controversial.

Methods

The authors studied serial samples of peripheral blood mononuclear cells (PBMC) from 11 patients with 25% to 95% surface area burns and 7 age-matched volunteer control subjects. Peripheral blood mononuclear cells were stimulated by the T-cell mitogen phytohemagglutinin (PHA), and IL-2 production and mRNA expression by Northern blot were determined. Expression and function of IL-2R were determined by monoclonal antibodies to the p55 and p75 chains of the IL-2R, binding of fluorescein-labeled IL-2, and response to exogenous recombinant IL-2. We also studied a mouse model of 20% burn injury known to mimic the immune abnormalities seen in humans with burns. Splenocytes from mice with burns (20–22 per group) were studied for IL-2 production and IL-2 mRNA expression after stimulation with the T-cell mitogen concanavalin A (ConA) and compared with sham burn control subjects. Kinetics of mRNA expression after ConA stimulation also were determined and a nuclear run-on assay performed to determine IL-2 gene transcription. The mRNA expression was determined for the proto-oncogenes *c-jun* and *c-fos,* whose protein products join to form transcription factor AP1, which is necessary for activation of the IL-2 promoter. Splenocytes from mice with burns after ConA stimulation also were studied for expression and function of the IL-2R.

Results

Peripheral blood mononuclear cells from burn patients compared with healthy control subjects showed diminished ($p < 0.05$) IL-2 production and mRNA expression 4 to 10 days after burn injury. Burn PBMC demonstrated normal expression of IL-2R, p55, and p75 chains 0 to 7, 8 to 20,

and 21 to 37 days after burn injury, normal IL-2R binding of fluorescein-labeled IL-2, and a normal proliferative response to PHA in the presence of exogenous recombinant IL-2. Splenocytes from mice 7 days after burn injury showed diminished production ($p < 0.05$) of IL-2 and IL-2 mRNA expression after ConA stimulation as compared with sham burn control subjects. Kinetics of mRNA expression after ConA stimulation were the same for burn and control mice, indicating that reduced IL-2 mRNA expression was not caused by altered mRNA degradation. A nuclear run-on assay confirmed decreased IL-2 gene transcription in burn splenocytes. Burn splenocytes showed normal expression of mRNA for *c-jun* but diminished expression of mRNA for *c-fos*. Finally, splenocytes from mice with burns after ConA stimulation showed normal expression and function of the IL-2R 7, 10, 14, and 21 days after burn injury.

Conclusions

These human and animal studies indicate that major burn injury depresses T-cell activation at the level of IL-2 gene transcription at least in part by inhibiting *c-fos* expression, whereas IL-2R expression and function remain normal and T-cell proliferation can be restored to normal levels by exogenous IL-2.

Serious traumatic and thermal injury has long been known to cause profound immunologic defects.[1] We, and others, previously have demonstrated that inadequate production of interleukin-2 (IL-2) is a fundamental component of immunosuppression in this setting.[2-4] Peripheral blood mononuclear cells (PBMC) from thermally injured patients have been shown to display a significantly decreased proliferative response to T-cell mitogens or antigenic stimulation.[4,5] Although decreased IL-2 production after thermal injury is undisputed, the status of IL-2 receptor (IL-2R) expression and activity in this setting is controversial. Previous studies have variously reported reduced[6] or increased[7] IL-2R p55 chain expression in patients who are severely burned. These studies looked only at cell-surface expression of the p55 protein without examining other parameters of IL-2R expression and function. Although methodologic differences may contribute partially to the apparent discrepancies between these studies,[7] currently, there is no clear answer as to the status of IL-2R in the setting of thermal injury.

Activation of T lymphocytes through the T-cell receptor (TCR) ordinarily is followed by the production of IL-2. The precise series of events leading from the TCR to the transcriptional activation of the IL-2 gene has been the subject of intense investigation. Binding of ligands to the TCR complex leads to activation of phospholipase C via regulatory G proteins. This is followed by hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating inositol tris-phosphate and diacylglycerol. These products, in turn, lead to mobilization of intracellular calcium and activation of protein kinase C (PKC), respectively. A series of poorly understood phosphorylation events follows, which culminates in transcription of the IL-2 gene. It is known that a promoter region exists, located in the 5′ flanking sequences of the IL-2 gene between −326 to −52 bp from the start of transcription.[8-10] This promoter contains binding sites for a number of transcription factors, all of which must be occupied for IL-2 transcription to occur.[8-11] Of these factors, AP-1 is thought to represent a major target of PKC activation.[12] It is encoded by the cellular proto-oncogene *c-jun,* whose product interacts with the protein product of *c-fos* through a leucine zipper, leading to a 300-fold increase in its binding activity to the IL-2 promoter.[13,14]

Previously, we have demonstrated that expression of mRNA for IL-2 is decreased after thermal injury in a murine model.[15] This decrease in mRNA expression is maximal 10 to 14 days after thermal injury. We also have shown that when the membrane-associated events are bypassed by stimulation of T cells with the combination of a phorbol ester and a calcium ionophore, the decrease in IL-2 in mRNA expression seen after thermal injury persists.[15] These studies suggest that the effect of thermal injury on IL-2 mRNA expression is downstream of the cell membrane signalling events leading to PKC activation. We have examined the effect of thermal injury on the intracellular processes distal to PKC activation. In particular, we have focused on determining whether decreased IL-2 mRNA expression was the result of pretranscriptional or post-transcriptional mechanisms and have examined the expression of the proto-oncogenes *c-fos* and *c-jun.*

We also have made a detailed investigation of both IL-2R expression and function in patients with major burns and in an animal model of thermal injury. We have examined the effect of thermal injury on expression of both the IL-2Rα and the IL-2Rβ chains and have assessed the effect of thermal injury on IL-2R function, through mea-

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surement of lymphocyte IL-2 binding and of the proliferative response to *in vitro* stimulation with exogenous IL-2.

MATERIALS & METHODS

Human Subjects

Eleven patients with major burns (25–95% body surface area) were studied after we obtained informed consent as approved by the Brigham and Women's Hospital Committee for the Protection of Human Subjects from Research Risks and in accordance with United States Public Health Service guidelines. Three of the patients were women, eight were men, and they ranged in age from 19 to 76 years, with a mean of 41 years. One patient died of his burn injury on day 28, and each individual had at least one episode of clinically diagnosed sepsis.

Twenty- to thirty milliliter-samples of venous blood were withdrawn at serial intervals from 2 to 28 days after burn injury. Peripheral blood mononuclear cells were isolated by centrifugation of heparinized blood on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) for 35 minutes at 400 g.[3] The interface cells were collected, washed three times in minimum essential medium (MEM) with antibiotics—2 mmol/L glutamine and 1% HEPES buffer and 5% fetal bovine serum (FBS)—and counted using trypan blue for viability and Turk's stain for observation of morphology. Cells were always more than 95% viable. Because patients' interface cells sometimes had increased contamination with myeloid cells, estimation of mononuclear cells by Turk's stain were made, and cell counts were adjusted accordingly so that the total number of mononuclear cells per well of all cultures was similar. Peripheral blood mononuclear cells were collected from one or more patients and one or two untreated healthy volunteers for each day's test.

Human PHA Stimulation: Measurement of Proliferative Response

Peripheral blood mononuclear cells, isolated in the aforementioned manner, were cultured in 220 μL/well volume at 1×10^5 cell/well in 96 well flat-bottomed tissue culture plates containing the aforementioned complete medium. Phytohemagglutinin (PHA) was added at a concentration of 6 μg/mL for 90 hours at 37 C in 5% CO_2, and the wells were pulsed with one μCi of tritiated thymidine (^{3}HTdr; New England Nuclear, Boston, MA) during the last 18 hours of culture. The cells were harvested on a multiautomated sample harvester (Cambridge Technology, Cambridge, MA), and incorporated radioactivity was measured in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). The mean amount of radioactivity was determined from triplicate cultures and expressed in counts per minute. Standard deviation of the mean of triplicates never exceeded 10%. The counts per minute (cpm) of the cultures containing no mitogen were subtracted from the cultures with mitogen, and this number was used in all calculations. Patients and healthy volunteers were compared.

Animal Model

One hundred forty male A/J mice, 7 to 8 weeks old, (Jackson Laboratories, Bar Harbor, Maine), each weighing 20 to 24 g, were acclimatized for a period of 1 week and randomized into two groups of ten mice each. After induction of anesthesia with intraperitoneal pentobarbital sodium (66 μg/g body weight), each mouse had its dorsum shaved. Then half were subjected to a 20% total body surface area, full-thickness scald burn; the remainder served as sham controls. Animals were resuscitated with 1 mL of 0.9% saline given subcutaneously and allowed water and mouse food *ad libitum*. All studies were performed with the approval of the Harvard Medical School Standing Committee on Animal Research and the National Institutes of Health. Animals were killed 4, 7, 10, 14, or 21 days after thermal or sham injury, and their spleens were harvested.

Splenocytes from each group were pooled and washed three times in RPMI 1640 with l-glutamine (2 mmol/1), HEPES buffer (10 mmol/1), 2-mercapto-ethanol (2-ME, 5×10^{-2} mmol/1; Eastman Kodak, Rochester, NY) and a 1% antibiotic/antimycotic solution containing 10,000 units of penicillin, 10 mg of streptomycin, and 25 μg of amphotericin B per milliliter. The above agents, unless otherwise stated, were obtained from Grand Island Biological Company (GIBCO), New York. Splenocytes were suspended again in the aforementioned medium with 5% heat-inactivated fetal calf serum (GIBCO) at a final concentration of 2×10^6 cells/mL and were incubated with or without ConA (Sigma Chemical Co., St Louis, Mo.) stimulation (2.5 μg/mL of cells). After 30 hours of stimulation, 1μCi of ^{3}HTdr was added to each well, and plates were rapidly frozen 18 hours later. Subsequently, plates were harvested using the multiautomated sample harvester, and ^{3}HTdr incorporation counted for 1 minute in the liquid scintillation counter. Mitogen responses were calculated by subtracting ^{3}HTdr incorporation in unstimulated cell cultures from that in mitogen-stimulated cultures.

Interleukin-2 Bioassay

Splenocytes from individual mice or PBMC from individual patients were cultured and stimulated with ConA or PHA, respectively, in the aforementioned man-

ner. At 48 or 24 hours, respectively, supernatants were harvested, and frozen at −20 C for assay later. Supernatants were diluted from 1:2 to 1:128 in 100 μL of complete medium, incubated for 1 hour at 37 C and 5% CO_2, and 100 μL of CTLL-2 cells were added. Cultures were incubated for 20 hours at 37 C in 5% CO_2, and proliferation was assessed by uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). Methylthiotetrazole was added (125 μg per well) and after 4 hours, cells were solubilized by the addition of 10% acidified sodium dodecyl sulfate (SDS). Uptake and conversion of methylthiotetrazole to formazan was determined in an automated enzyme-linked immunosorbent assay reader (Molecular Devices, Mountain View, CA) at 570 nm, using 650 nm as reference. Interleukin-2 production was calculated from standard curves using probit analysis[3] (software provided by Brian Davis of Immunex Corp, Seattle, WA).

Interleukin-2 Receptor p55 Protein Expression in CD3+ Cells

Peripheral blood mononuclear cells from patients who were burned or mouse splenocytes were harvested and cultured in the presence of PHA or ConA for 48 hours, after which viability was assessed with trypan blue stain. One million viable cells were incubated for 45 minutes at 4 C in the presence of fluorescein-conjugated monoclonal anti-CD3 antibody (Boehringer Mannheim, Indianapolis, IN) and phycoerythrin-conjugated anti-IL-2R p55 chain or p75 chain antibody (Boehringer Mannheim) and then fixed in 1% paraformaldehyde in phosphate-buffered saline. Samples were processed using an Epics C flow cytometer with a single 488-nm argon laser, counting 5000 cells per sample. Forward and 90° two-color analysis was used, and the data were processed by computer using Howard Shapiro 4cyte software. Nonspecific fluorescence and background staining was accounted for using appropriate controls, including normal murine Immunoglobulin G and staining of unstimulated cells.

Cellular Interleukin-2 Binding

Human PBMC or mouse splenocytes were stimulated with PHA or ConA for 48 hours, after which 10^6 viable cells were incubated for 1 hour at 4 C with biotinylated recombinant human IL-2 (Boerhinger Mannheim), then washed and incubated for 45 minutes in the presence of an avidin-fluorescein complex. Samples were evaluated again by flow cytometric analysis, as aforementioned. Nonspecific fluorescence and background staining were accounted for using an irrelevant first-step antibody, followed by the same second step reagent. As a further test of IL-2R function, PBMC from 9 patients with burn injuries who were significantly depressed in their response to PHA on 12 occasions ranging from 2 to 27 days after burn were cultured with PHA as above in the presence of 10 and 100 units/mL of human recombinant IL-2 (hurIL-2) (Amgen, Thousand Oaks, CA) and tested for proliferation. Similarly, splenocytes from mice that were burned were cultured with ConA in the presence of hurIL-2 4, 7, 10, 14, and 21 days after burn injury.

cDNA Probes

The following cDNA probes were used in this study. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β actin, human and murine IL-2 probes were purchased from the American Type Culture Collection, Rockville, Maryland. *C-fos* and *c-jun* probes were provided by Dr. A Lichtman of Harvard Medical School and Brigham and Women's Hospital. Probes were radiolabelled using a random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, IN).

Northern Blotting

After stimulation *in vitro* for 2 to 24 hours, human PBMC or mouse splenocytes were lysed in guanidium isothiocyanate and 2-ME. Total RNA was isolated by phenol/chloroform extraction. Equal quantities of RNA (5 μg/lane) were fractionated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to nylon membrane, and fixed by ultraviolet cross-linking. Membranes then were hybridized for 18 hours at 42 C with the [α-^{32}P]dCTP (New England Nuclear Research Products, Boston MA)-labeled cDNA probe for human IL-2 or murine IL-2, *c-fos* or *c-jun*. Then blots were washed four times with SSC/SDS solution and autoradiographed. Blots were stripped at 80 C using a 1% glycerol solution (Sigma Chemical Co., St Louis, MO) and probed again with a radiolabelled cDNA probe for GAPDH. Signals were quantitated using a laser densitometer (Molecular Dynamics, Model 300 Series, Waltham, MA) and were standardized to the GAPDH message.

Transcriptional Rate Assay

Nuclear transcriptional rate assay was performed as described by Chen.[16] Briefly, cultured mouse splenocytes were harvested and washed three times in ice cold phosphate-buffered saline. Nuclei then were isolated at 0 C by resuspending cells in buffer A (glycerol, Tris-HCl pH7.9, $MgCl_2$ and Triton × 100). The lysate was homogenized and pelleted through a 30% sucrose cushion. The nuclear pellet was suspended again in suspension buffer (glycerol, Tris HCl ph 8.3, $MgCl_2$ and edetic acid [EDTA]),

adjusted to 3.6×10^8 nuclei/mL, and rapidly frozen in liquid nitrogen. One hundred microliters of nuclei from both sham and burn groups were thawed subsequently. Nascent transcripts were elongated by incubating each sample with transcription reaction mix containing Uridine 5′-[α-^{32}P]triphosphate (New England Nuclear Research Products). The mixture was incubated and the RNA was subsequently extracted twice with phenol/chloroform (1:1) and twice with chloroform. The extract was incubated with trichloroacetic acid-60mM pyrophosphate for 30 minutes on ice and then filtered onto nitrocellulose filters (0.2 μm pore, Schleicher & Schuell, Keene, NH) with the aid of a vacuum. The filters were washed and RNA was extracted twice with phenol/chloroform and once with chloroform. After centrifugation (1500 g $\times$ 30 min), the RNA pellet was again suspended in 140 μL of Tris-EDTA buffer, heated to 65 C for 10 minutes, and cooled on ice. A 2 μL-aliquot of each sample was trichloroacetic acid-precipitated and subjected to scintillation counting to determine the incorporation of radionucleotides. Equal counts per minute of RNA from sham and burned mice then were hybridized, at 42 C for 72 hours, with strips of nylon membrane on which equal amounts of the cDNA probe for murine IL-2 or GAPDH had been immobilized by Southern blotting. The membranes then were washed with salt sodium phosphate edetic acid (SSPE)-0.1% (vol/vol) SDS and autoradiographed.

Statistical Analysis

Control subjects *versus* burn patients and sham burn mice *versus* mice with burns were compared, and results were expressed as percentage change in the burn group compared with the sham group. Proliferation, cytokine production, IL-2R expression, and IL-2 binding were compared between the different groups using the Mann-Whitney U test or Student's t test. Results were considered significant if $p \leq 0.05$.

RESULTS

T-Lymphocyte Proliferation and IL-2 Production

The proliferative response of PBMC from burn patients to mitogen stimulation was significantly suppressed as compared with age-matched control subjects 4 to 10 days after burn injury; this was also true for the IL-2 production (Table 1).

The response of mouse splenocytes in response to ConA stimulation in mice with burns was suppressed consistently in comparison with control animals, maximally by 50% ($p < 0.05$) on day 4. Interleukin-2 production in ConA-stimulated burn mouse lymphocytes was similarly suppressed, maximally by 40% ($p < 0.05$) on day 7.

Table 1. BURN PATIENT PHA RESPONSES—DAYS 4–10 POST-BURN

	Patients (n = 6)	Controls (n = 6)
PBMC proliferation (cpm ± S.E.)	29,680 ± 13,458*	108,570 ± 27,152
PBMC IL-2 production (U/mL ± S.E.)	0.01 ± 0*	0.20 ± 0.01

* $p < 0.05$.

IL-2R Expression

Cell surface IL-2R p55 chain or p75 expression in mitogen-stimulated $CD3^+$ cells was not changed in the burn patients compared with the age-matched control subjects at any day measured. Figure 1 displays the results of 19 observations made in seven patients at varying intervals after burn injury. The percentage of $CD3^+$ cells expressing IL-2R p55 varied between 50% and 80%, and at any given time, results in burn and control groups were very similar. The variation in p75 chain expression was somewhat greater, tending to be higher in burn patients than in control subjects, but again, there was no significant difference.

No significant difference in p55 chain expression was noted in burn mouse splenocytes on any day after injury as compared with sham burn splenocytes. Expressed as per cent difference from sham burn, burn splenocytes varied from +9% on day 7 to −5% on day 21 after burn injury.

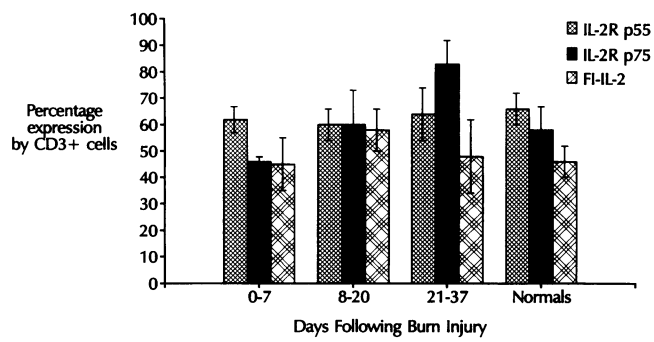

Figure 1. Histogram showing the expression of the IL-2R p55 and p75 chains on PBMC from burn patients at serial intervals after burn injury and from normal control subjects. Binding of fluoresceinated IL-2 also is shown. Results are presented as mean percentage of CD3+ cells binding the appropriate antibody ± SE.

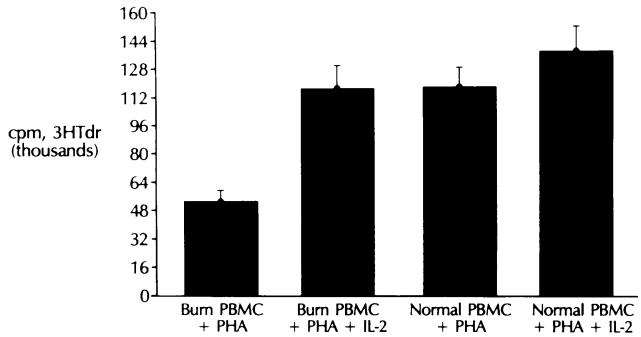

Figure 2. Histogram demonstrating the effect of the addition of exogenous rhuIL-2 at 10 units or 100 units/mL to PHA-stimulated PBMC cultures from nine burn patients whose PHA responses were significantly suppressed on 12 occasions, compared with simultaneously studied normal control subjects from 2–37 days after burn. The effect of exogenous rhuIL-2 on PHA-stimulated cultures of PBMC from normal individuals simultaneously studied also is shown. Results are expressed as mean cpm ± SE.

IL-2 Receptor Functional Studies

Binding of fluorescein-labelled IL-2 was unchanged in burn patients compared with volunteer control subjects at any interval after injury (Fig. 1). Compared with sham burn mice, animals with burns showed no decreased IL-2 binding 4, 7, 10, 14, and 21 days after burn injury. Expressed as per cent difference from sham burn, burn splenocytes varied from +117% on day 10 to −7% on day 21.

Addition of IL-2 at 10 units and 100 units/mL to mitogen-stimulated cultures of PBMC from burn patients (Fig. 2) or splenocytes from mice with burns caused a significant increase in the proliferative response to mitogens on all days measured after injury. One of the two IL-2 concentrations restored the mitogen response to normal levels in all instances, indicating the presence of functional cellular IL-2 receptors.

IL-2 mRNA Expression

We found expression of mRNA for IL-2 to be markedly decreased in PBMC from two burn patients compared with healthy control subjects 7 days after injury (Fig. 3). Consistent with our previous findings,[15] we also found expression of mRNA for IL-2 in murine splenocytes 7 days after thermal injury to be significantly decreased compared with sham burn control subjects (Fig. 4).

Then we investigated the possibility that this decrease was caused by a temporal alteration in the expression of mRNA for IL-2 after thermal injury. Therefore, we examined the kinetics of expression of IL-2 mRNA in both sham mice and mice with thermal injury. Figure 5 demonstrates the expression of IL-2 mRNA, which is maximal between 12 and 24 hours after *in vitro* stimulation in both sham and burn groups. These data show that there was no alteration in the kinetics of IL-2 mRNA expression after thermal injury.

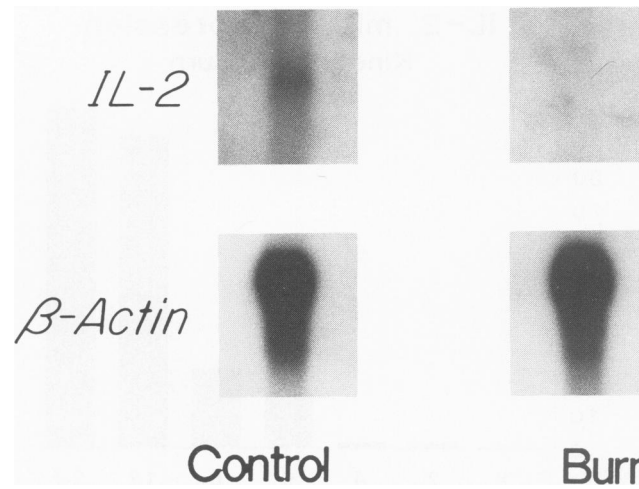

Figure 3. Northern blot of RNA extracted at 6 hours from PHA-stimulated PBMC of a patient who was burned (right lane) 7 days after injury and from a simultaneously studied normal control subject (left lane). Expression of the IL-2 message is diminished in the burn patient. Control β actin message expression also is shown.

IL-2 Transcriptional Rate Assay

Having established that the expression of IL-2 mRNA was decreased after thermal injury, we faced two possible explanations. First, the decreased mRNA expression was the result of a decrease in the stability of newly transcribed mRNA, leading to an increase in its metabolism after thermal injury. Second, and more likely, thermal

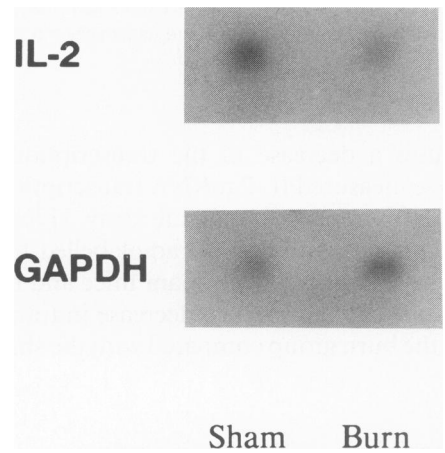

Figure 4. Northern blot of RNA extracted from spleens of burn and sham burn mice 7 days after burn. Expression of the message for IL-2 is diminished in the burn splenocytes. GAPDH message also is shown.

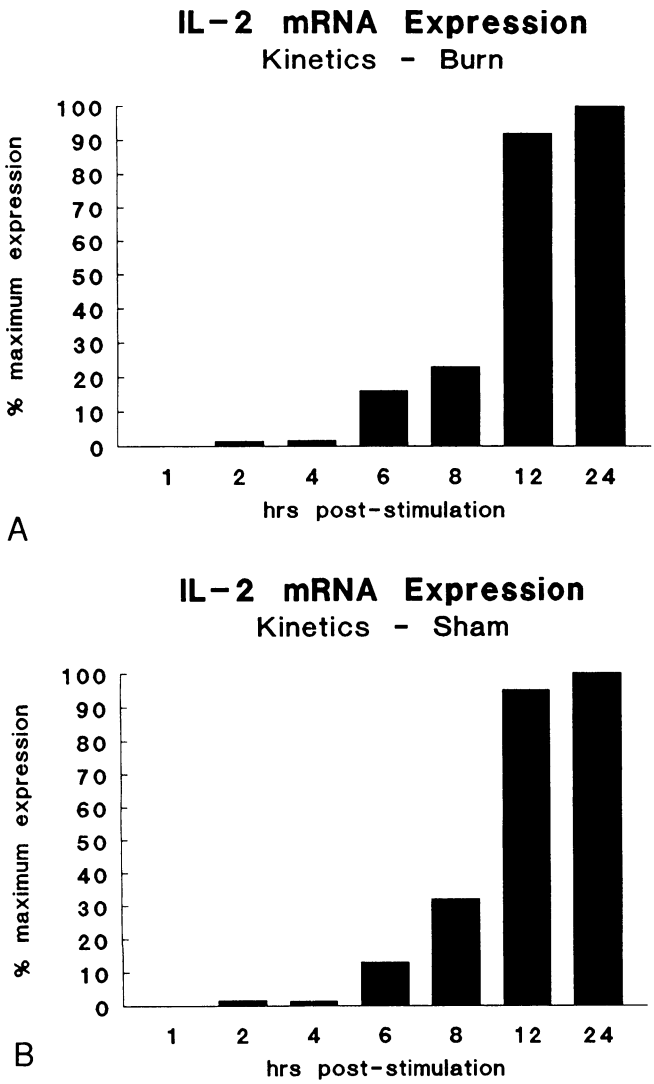

Figure 5. (A) Kinetics of splenocyte IL-2 mRNA expression 7 days after burn injury as determined by laser densitometry of Northern blots. Results are expressed as a percentage of maximum mRNA expression after *in vitro* stimulation. (B) Splenocyte IL-2 mRNA expression 7 days after sham burn. The kinetics of mRNA expression are essentially the same in both groups.

injury causes a decrease in the transcription of IL-2 mRNA. We measured IL-2 mRNA transcription using a nuclear run-on transcriptional rate assay. Hybridization of equal counts per minute of radiolabelled transcripts from the isolated nuclei from sham mice and mice with burns demonstrated a marked decrease in transcription of IL-2 in the burn group compared with the sham group (Fig. 6).

Proto-oncogene mRNA Expression

To further elucidate the mechanisms underlying decreased IL-2 mRNA transcription, we focused our attention on expression of the *c-fos* and *c-jun* genes. Expression of mRNA for *c-fos* was found to be maximal after 30 minutes of stimulation with ConA in both sham mice and mice with burns and was not detectable after 2 hours of stimulation. Expression of mRNA for *c-fos* was markedly decreased in the group with thermal injury compared with the sham group (Fig. 7). Expression of mRNA for *c-jun* became detectable after 6 hours of *in vitro* stimulation and was still present as late as 24 hours after stimulation. In contrast to *c-fos*, thermal injury did not alter the expression of *c-jun*.

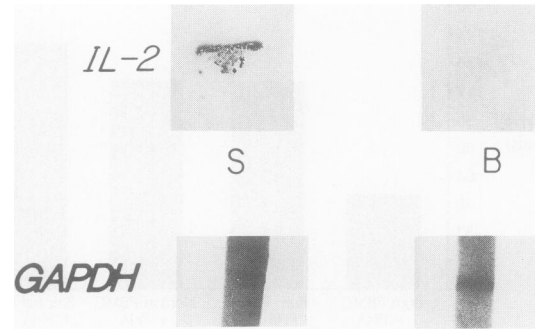

Figure 6. Nuclear transcriptional rate assay performed on splenocytes from burn and sham burn mice on day 7. Transcription of the IL-2 message in the burn splenocytes is markedly reduced. Transcription of the GAPDH message is shown as a control.

DISCUSSION

Decreased T-cell production of IL-2 plays a fundamental role in the immunosuppression seen after thermal injury.[2-4] The intracellular events leading to this inadequate IL-2 production after thermal injury, however, have only begun to be elucidated. Our knowledge of intracellular signal transduction, particularly in T lympho-

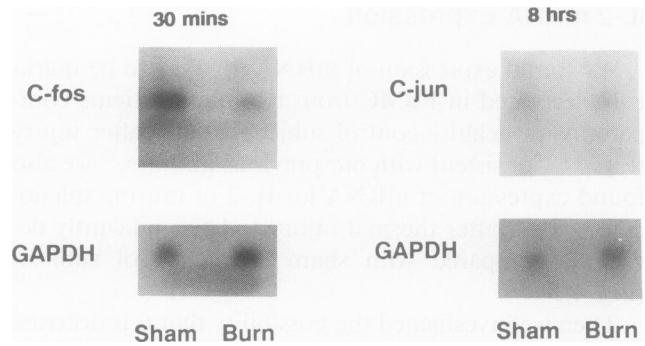

Figure 7. Northern blots of splenocyte RNA from burn and sham burn mice 7 days after burn injury probed for *c-fos* and *c-jun* message. It is apparent that *c-fos* expression is markedly reduced in burn splenocytes, whereas *c-jun* expression is not significantly different from sham burn splenocytes. The GAPDH message is shown as a control.

cytes, recently has increased dramatically. However, abnormalities of signalling pathways in the immunocompromised patient have yet to be delineated. Having demonstrated that IL-2 mRNA expression is decreased after thermal injury in patients and in a murine model, we investigated whether thermal injury alters IL-2 mRNA expression by a pre- or post-transcriptional mechanism. Post-transcriptional regulation could result from altered stability of the mRNA or from an increase in the rate of its degradation by induction of RNases.[15,16] There was no difference in kinetics of IL-2 mRNA expression between burn and control groups, suggesting that simple temporal differences in IL-2 message expression did not account for our findings. Nuclear run-on transcriptional rate assay demonstrated reduced IL-2 mRNA transcription in animals that were burned, indicating that the changed mRNA expression was occurring pretranscriptionally.

Then we focused on the pretranscriptional nuclear components of the signal transduction pathway and examined mRNA expression for the cellular proto-oncogenes *c-fos* and *c-jun,* the protein products of which dimerize via a leucine zipper to form the nuclear binding protein AP-1. The transcriptional activation protein AP-1 is a major target of activated PKC and is essential for IL-2 gene transcription.[12] Furthermore, fos and jun proteins recently have been shown to be components of the transcriptional factor NFAT in both murine[19] and human[20] T cells. *C-fos* and *c-jun* appear to be regulated through disparate intracellular pathways. Peripheral blood lymphocytes have been shown to constitutively express mRNA for *c-jun.*[20] However, *c-fos* is undetectable in resting PBMCs, and levels increase dramatically after PHA stimulation. The proto-oncogene *c-fos* is induced maximally within 30 minutes of T-cell activation, with transcripts becoming apparent as early as 5 minutes after stimulation. Expression of *c-fos* mRNA rapidly declines thereafter because of transcription repression by its own product, the fos protein.[21] Our studies showed that after thermal injury, the expression of mRNA for *c-fos* was markedly reduced compared with sham control subjects. In contrast, *c-jun* mRNA levels were unaltered in splenocytes from the animals with thermal injury. It has been suggested that binding of the jun protein to the enhancer region of the IL-2 gene in the absence of *c-fos* product may act as a negative regulator of transcription; initiation of transcription will not occur until levels of fos protein increase.[20] Therefore, mitogenic or antigenic stimulation of the TCR complex and appropriate accessory receptors will lead to full activation and binding of the AP-1 complex to the IL-2 enhancer region and, presumably, to transcriptional activation of the IL-2 gene. Our finding of decreased levels of mRNA for *c-fos* after thermal injury suggests that it is through this signalling pathway, leading from the TCR to transcription of *c-fos,* that thermal injury exerts its negative influence on IL-2 production. This is consistent with the findings reported by Introna et al.,[22] who demonstrated a desensitization of *c-fos* to further stimulation after initial activation of murine peritoneal macrophages with endotoxin. This occurred despite normal levels of PKC activity and was evident at the level of *c-fos* transcription.

The current findings, taken in conjunction with previous studies, [15,23] suggest that an important component of the intracellular signalling defect leading to the decrease in production of IL-2 lies between PKC activation and *c-fos* transcription. The factors that influence the transcription of *c-fos* after thermal injury will require further investigation in the hope that localization of the defect will lead to therapeutic measures aimed at reversing or averting the associated immunosuppression.

Expression of functional high affinity IL-2 receptors also is critical to the normal lymphocyte response to antigenic or mitogenic stimulation and is linked closely to expression of the IL-2Rα (p55) gene product. Other groups previously have addressed the question of IL-2Rα expression after trauma or thermal injury. Teodorczyk-Injeyan and colleagues[6] have shown a decrease in the percentage of T lymphocytes that express IL-2Rα in patients after thermal injury. In these studies, marked cellular IL-2Rα suppression was observed in very severe, terminal burn cases; however, in patients injured less severely, IL-2Rα suppression was less severe and transient.

There is considerable evidence to dispute depressed cellular IL-2R expression after thermal injury. Schluter and colleagues recently reported increased IL-2Rα expression in patients that were severely burned.[7] A number of studies have suggested the presence of functional cellular IL-2R by demonstrating a significant proliferative response of lymphocytes from burn victims, with the addition of recombinant IL-2 to *in vitro* cultures.[24] This study attempted to resolve this controversy by serial observations of IL-2R expression and function in patients with major burns and in an animal model of burn-induced immunosuppression.

Our results demonstrate normal expression of cell surface IL-2Rα and IL-2Rβ in mitogen-stimulated, $CD3^+$ cells after burn injury in humans. Interleukin-2 receptor expression also was never reduced at any interval in the mouse with burns. Additionally, we demonstrated that IL-2R function is normal after this degree of thermal injury. Cellular binding of IL-2 was normal early and late after injury. The addition of human recombinant IL-2 to *in vitro* cell cultures restored the proliferative response of suppressed lymphocytes to normal, demonstrating the presence of functional IL-2R.

It makes intuitive sense that IL-2R is unaffected, but IL-2 production is reduced, because the signals required

for IL-2R gene expression appear to be considerably less specific and less stringent than those required for IL-2 gene expression. A number of papers have explored differences in IL-2 and IL-2R induction, and may provide clues to the mechanism of dissociated IL-2 and IL-2R expression in our studies. Stimulation of the TCR/CD3 complex by CD3 antibodies gives rise to low-level stimulation of PKC and can induce IL-2 receptor expression, but the failure of this stimulus to induce IL-2 production suggests that higher signalling thresholds exist in the latter case.[25-27]

Prostaglandin E_2 (PGE_2) release from macrophages is induced after thermal injury[24] and results in suppression of T-cell function, most likely mediated through the intracellular messenger, cAMP. Although it has been shown that increased PGE_2 production and cAMP concentration decrease lymphocyte proliferation, IL-2 production, and expression of high affinity IL-2R,[28-31] the effect on IL-2Rα expression is controversial. Some investigators have shown down-regulation of IL-2Rα by cAMP,[28-30] whereas others have shown normal or increased IL-2Rα expression with increased cAMP.[31-32] Exactly where these data fit with our present experiment that shows normal IL-2Rα expression in the presence of presumed elevations of PGE_2 and cAMP, is unclear. The jump from *in vitro* experimental results to the *in vivo* situation is a large and uncertain one. We cannot make the assumption that because addition of cAMP analogues to *in vitro* cell cultures inhibits IL-2Rα expression, the same applies to *in vivo* cellular exposure to more physiologic concentrations of PGE_2. It is quite possible that at physiologic concentrations, PGE_2 has a strong enough inhibitory effect to reduce IL-2 production without having an appreciable effect on IL-2R expression.

The current studies provide strong evidence for normal IL-2R expression and function in the presence of suppressed lymphocyte activation. Because IL-2 and IL-2R are the chief components of the T-cell proliferative response to antigenic stimulation, the suppression of T-cell proliferation that follows severe thermal injury or trauma appears primarily related to abnormal IL-2 production.

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Discussion

DR. BASIL A. PRUITT, JR. (Fort Sam Houston, Texas): It's a privilege to discuss this paper by Dr. Mannick and his colleagues, who have presented both human and murine model data confirming and extending their earlier studies of the immunosuppressive effects of burn injury.

They have identified a transcriptional deficit as manifested by marked impairment of monocyte expression of the c-fos proto-oncogene. It is tempting to relate this defect to the burn injury per se. In that regard I would ask whether the heterogeneity of burn size and of age in the patients permitted one to correlate those variables with the changes observed? In that same vein, are the changes in the murine model burn-size related? That is, were the changes reduced in magnitude in animals with 10 or 15% burns, or conversely, since you can't generate a larger burn in a surviving mouse model, have you evaluated these changes in a rat model with a larger burn, 30, 40, or 50%?

Additionally, Dr. Mannick noted that all 11 patients had at least one infection. Consequently, one wonders whether the observed changes were caused or exaggerated by infection.

Dr. Mannick and his group have shown that certain aspects of the metabolic response to injury can be mimicked by hormonal infusions. Accordingly, I wonder whether the immunosuppression you have observed in these studies can be correlated with circulating levels of steroids or catecholamines in either the patients or the mice and whether such changes can be evoked by neurohormonal manipulation without burning.

Lastly, is it possible by genetic engineering to correct the defect in c-fos expression or alternatively provide additional transcription factor AP-1?

DR. RONALD V. MAIER (Seattle, Washington): It is truly a privilege to be asked to discuss this excellent paper. As Dr. Pruitt mentioned, it's a very logical and elegant continuation based on the previous findings by this excellent laboratory.

Basically, the decrease in response of T-cells following burn injury is linked directly to decreased production of IL-2 and decreased production of IL-2 is due to decreased transcription of messenger RNA for IL-2. Moving through the molecular steps to determine the mechanism, they have shown that there appears to be a decreased transcription of the c-fos messenger RNA which is obligatory for the induction of IL-2 transcription.

Several questions arise from these studies which I'd like to ask the authors. In prior experiments the authors showed that direct activation of PKC did not reverse the T-cell inhibition. In this paper, and other recent publications, looking at the activation of other genes dependent upon the AP-1 nuclear factor, it has been shown that PKC is effective in activating c-fos and c-jun and thus generating AP-1 which then stimulates the target gene transcription. If the current hypothesis is correct, why in their preliminary experiments did the authors not see reversal of the inhibition when they directly activated PKC?

Is the possible argument therefore that PKC may be necessary but not sufficient? That is, does activation of c-fos occur rapidly due to other activation pathways? It's been proposed that certain receptors, such as the T-cell receptor, may structurally be bound to tyrosine kinases which can directly activate the c-fos pathway without going through PKC. Do the authors have evidence for this or can they support this conjecture?

Secondly, the cells have been co-incubated with monocytes in their human studies. As the authors present in their manuscript, it's known that macrophages after burn injury increase their production of PGE-2. PGE-2 increases cyclic AMP, and concomitantly cyclic AMP-dependent kinase as a mechanism of down-regulating the inflammatory response. This is undoubtedly operative in their experimental scenario.

Do the authors have any data that the effect they are seeing is due to PGE-2 production by the co-cultured monocytes? Can they block this effect with antibody to PGE-2 or by inhibiting PGE-2 production? If they isolate the T-cells and incubate them, can they reverse the defect by removing the influence of PGE-2? Is it PGE-2? Is it IL-10, that is also known to be produced in large amounts by monocytes in this setting and shown to downregulate T-cell function?

Finally, while the authors use as a functional endpoint proliferation of the T-cell, which is undoubtedly important in the host immune system, do they have any other data to support that these effects have an impact in vivo? That is, if one reverses this T-cell defect of IL-2 production by giving exogenous IL-2,